Examining the role of Deltalike3 in Notch Signaling during Vertebrate Segmentation

A Senior Honors Thesis

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by

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Table of Contents

I. Introduction

p. 3-22

II. Results

p. 22-34

III. Discussion

p. 35-39

IV. Materials and Methods

p. 39-42

V. References

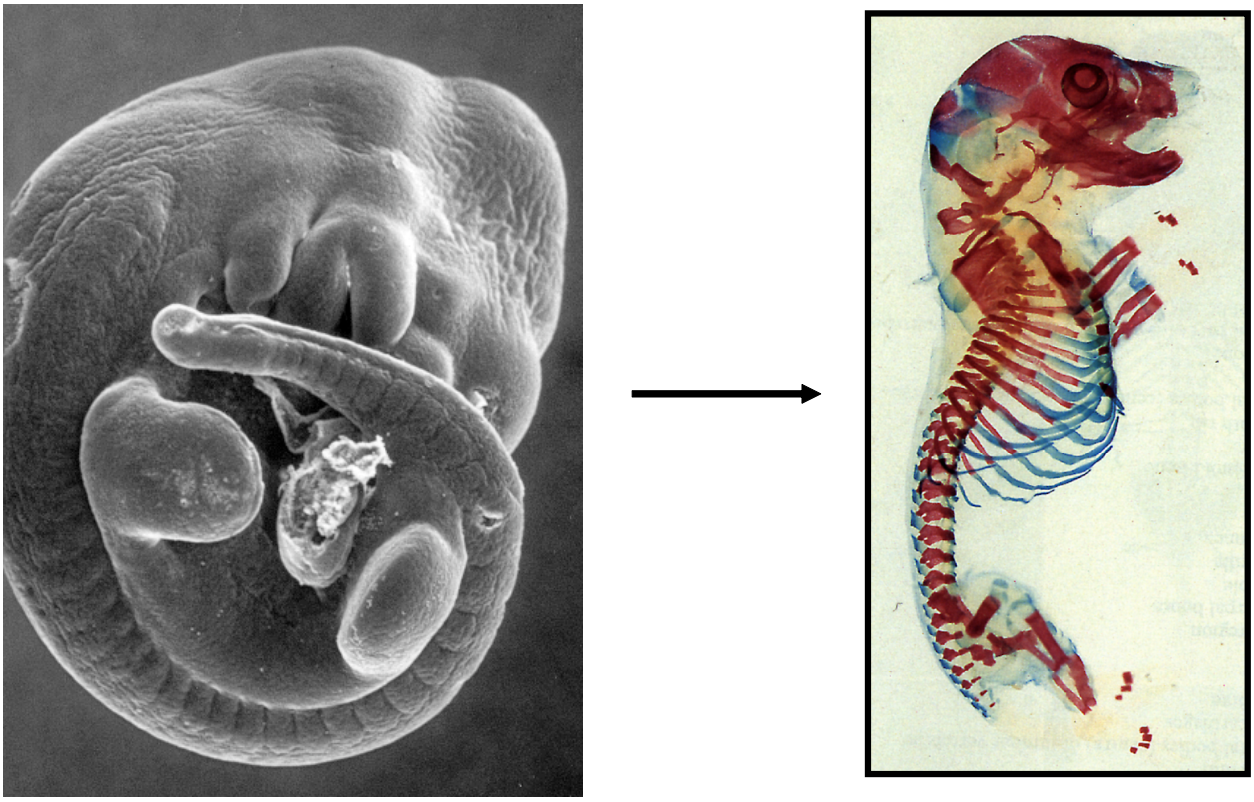
p. 43-44

I. Introduction

Vertebrae segmentation is an embryological process regulated in part by the Notch signaling pathway. The unperturbed temporal and spatial activities of the genes involved in the Notch signaling pathway are responsible for proper skeletal phenotypes of vertebrates. The activity of Deltalike3 (*Dll3)*, a Notch family member has been suggested to be important in both the clock and patterning activities of the Notch signaling pathway. However, the importance of *Dll3* in the clock or patterning activities of the Notch signaling for proper segmentation events to occur has not been examined. Loss of Deltalike3 expression or activity in mice results in severe vertebral abnormalities, which resemble the phenotype of mice that lack the gene Lunatic fringe (*Lfng)*, proposed to be an inhibitor of Notch. Despite the phenotypic evidence suggesting that *Dll3* is an inhibitor of Notch like *Lfng*, there is other conflicting data suggesting that *Dll3* may act either as an inhibitor or activator of Notch. My project intends to examine the role of *Dll3* as an inhibitor or activator of Notch, to determine whether the *Dll3* has a more important role in the clock or patterning activities of Notch signaling, and to analyze the possibility for modifier effects between *Dll3* and other Notch family members.

III. Somitogenesis Overview

The characteristic re-iterated skeletal pattern of vertebrates is determined during an embryological process called somitogenesis. During this process, somites, consisting of blocks of mensenchymal tissue, bud from the presomitic mesoderm (PSM), an overtly unsegmented tissue. These somites are formed in a specific spatial and temporal manner, and are the precursors for segmented structures in the organism such as the vertebral column and ribs (Fig 1) (reviewed in Shifley et al. 2007). The somites have rostral and caudal compartments, which contribute to the metameric pattern of somatic derivatives. For example, the rostral and caudal



(courtesy of Tom Vogt) **(Kaufmann, 1992)**

Figure 1. Somitogenesis, an embryological process, is responsible for the re-iterated patterns of the vertebrate skeleton. The scanning electron microscope image (left) shows a mouse embryo at 12.5 d.p.c. The mouse skeleton at 17.5 d.p.c. (right) is stained with alizarin red and alcian blue for visualization.

compartments delineate the adult boundaries of structures, so that an adult vertebrae arises from the fusion of the rostral half of one sclerotome and the caudal half of another (Fig. 2) (reviewed in Dubrulle et al. 2004).

III. Complex regultation of somitogenesis

During the formation of somites, the PSM exhibits patterning and clock activities. Within the posterior region of the PSM (region I) a segmentation clock functions to time somitogenesis. This is characterized by having oscillatory mRNA expression of numerous genes which cycle with a period identical to the rate of somite formation. In the anterior region of the PSM, many cyclic genes become stabilized. The stripe of stable mRNA expression in the anterior PSM reflects the rostral/caudal (R/C) patterning of a future somite. The rostral/caudal identity of the pre-somite is established in the anterior region of the PSM. Thus, the anterior PSM contains pre-somites and the posterior PSM is unsegmented (Fig. 3, 4) (reviewed in Dubrulle et al. 2004).

The clock in the posterior PSM is believed to interact with positional information encoded by a wavefront, which is established by a gradient of fibroblast growth factor (FGF) extending from the posterior region of the PSM. This wavefront defines positional information coordinating when cells are ready to form a somite, with the most mature cells being located in the anterior-most PSM where there is a lower concentration of FGF. The clock is regulated by the timely activation and deactivation of the Notch signaling pathway in the posterior PSM. Thus, the clock allows for pulses of Notch expression which are required for the reiterated pattern of somites in the anterior skeleton (reviewed in Weinmaster et al. 2003).

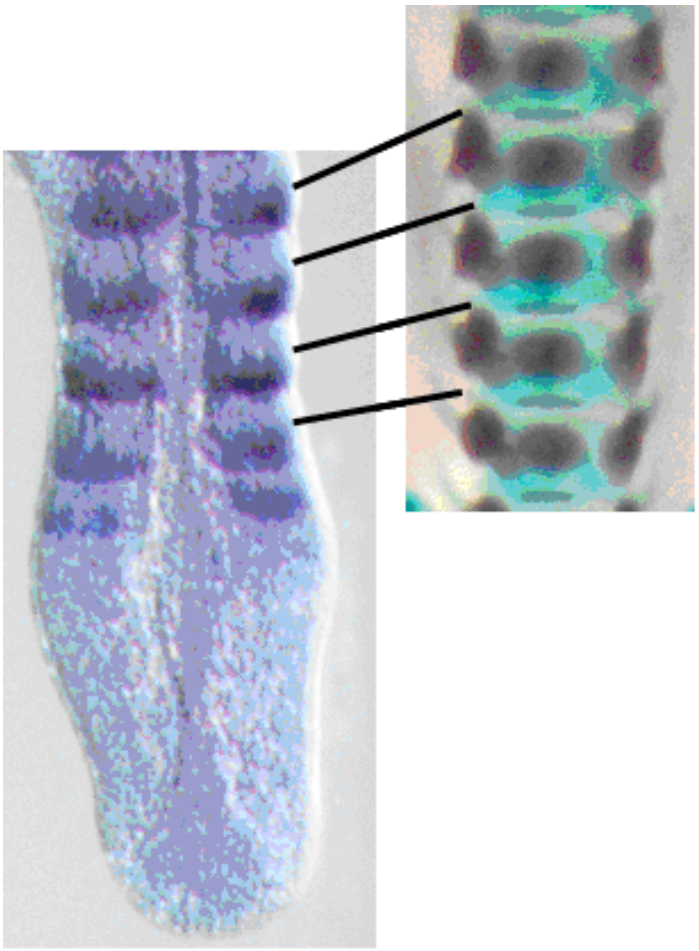


Figure 2. Resegmentation. An adult vertebrae arises from the fusion of the rostral half of one sclerotome with the caudal half of another. The left panel *in situ* image shows the caudal delineation of somites with the Uncx4.1 RNA probe in 10.5 d.p.c. mouse embryos. The right panel shows the vertebrae of a 17.5 d.p.c. mouse skeleton stained with alizarin red and alcian blue for visualization.

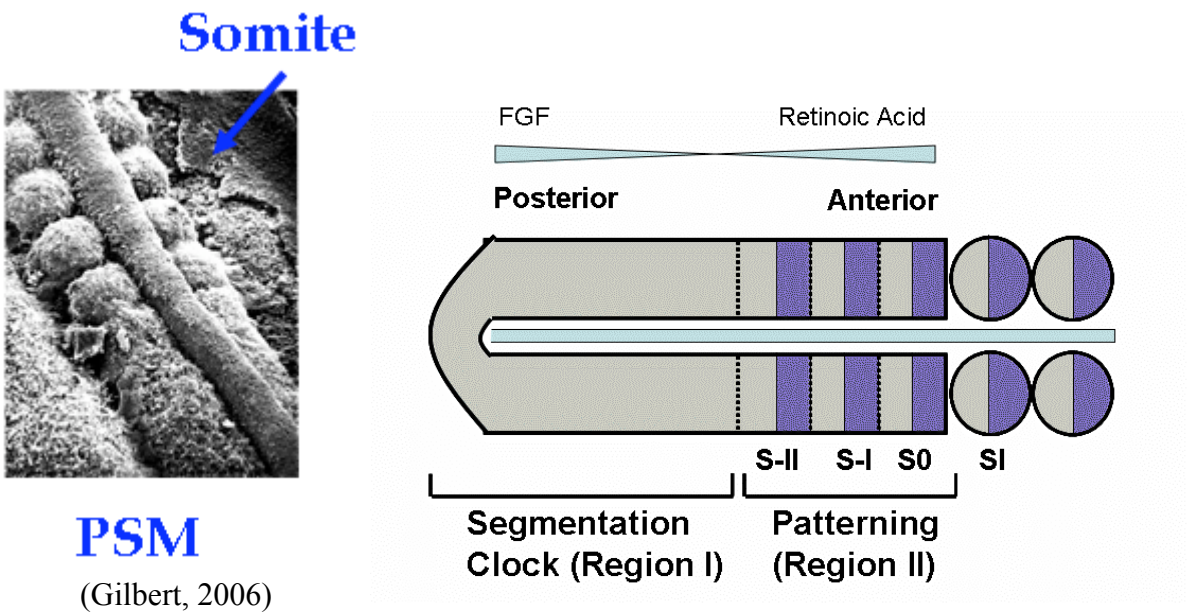


Figure 3. Somitogenesis, Clock/Patterning. The scanning electron micrograph image (left) depicts somites in a chick embryo. The schematic (right) represents the pre-somitic mesoderm and somites, and it explains the events of somitogenesis. Somites bud from the PSM, becoming precursors for the segmented structures of the vertebrate and for skeletal muscle. The segmentation clock is active in the posterior PSM (Region I). In the anterior PSM (Region II), presomites are patterned.

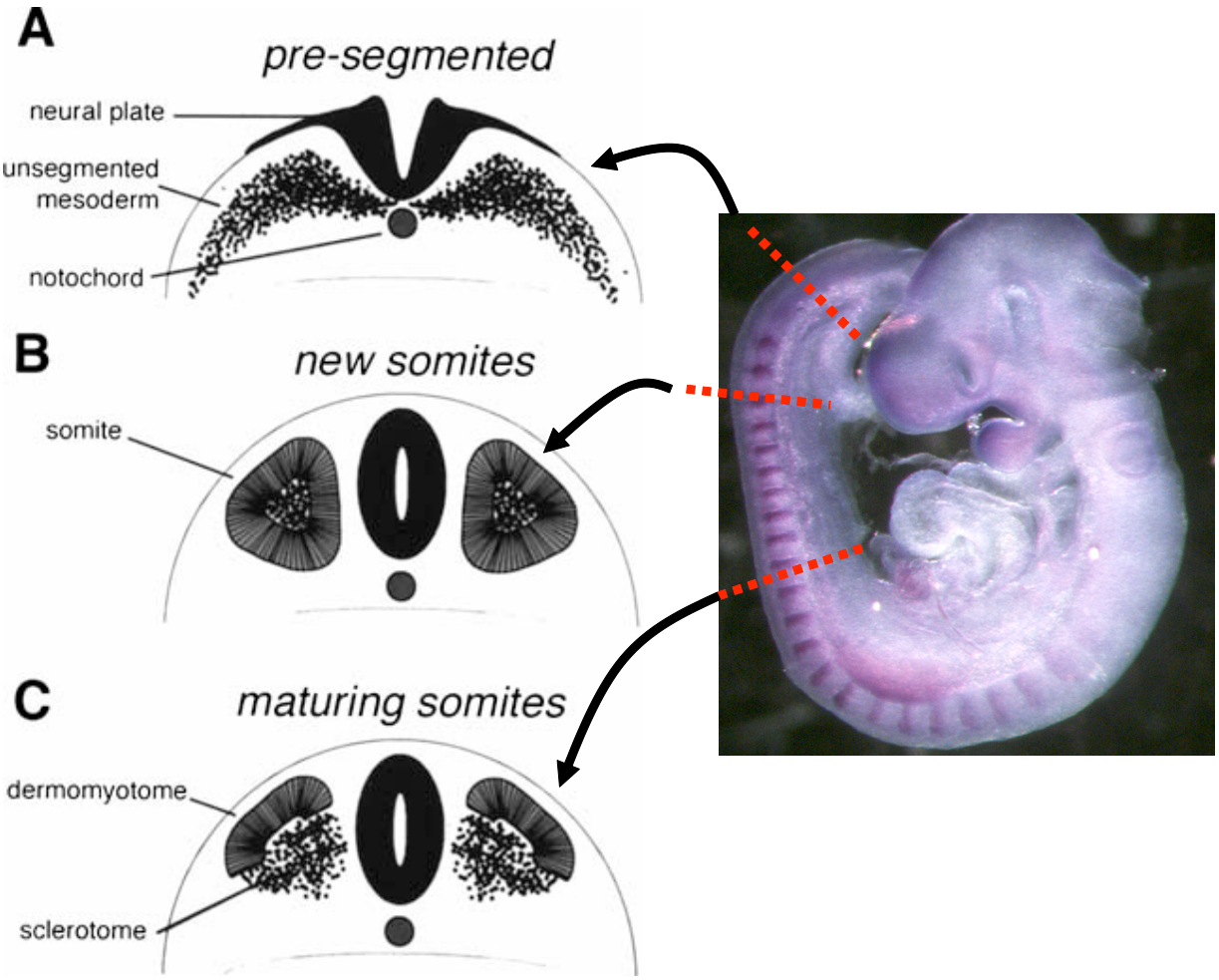


Figure 4. Maturing somites during vertebrate segmentation. The image on the left represents the changes that occur as somites mature. The image on the right is an *in situ* image of a mouse embryo at 9.5 d.p.c. The Uncx4.1 probe used delineates caudal compartments of somites.

IV. Notch Signaling Mechanism.

Notch signaling is activated when ligands of the Delta, Serrate and LAG2 families (DSL) bind to a Notch receptor. In mammalian cells, it appears that DSL binding in the absence of endocytosis does not result in Notch receptor heterodimer dissociation. Therefore, it has been proposed that the DSL ligand endocytosis generates a force that removes Notch extracellular domain (NECD). In the Notch cell, the Notch intracellular domain (NICD) undergoes proteolytic activation by ADAM and γ–secretase cleavages (reviewed in Nichols et. al. 2007). Soluble active NICD then translocates to the nucleus where it functions as a transcriptional coactivator of CBF1 target genes, such as HES and fringe genes (Fig. 5) (reviewed in Weinmaster et. al. 2003).

The Notch receptor family members have epidermal growth factor (EGF)-like repeats within the extracellular ligand-binding domain. This region is important for some ligand-receptor interactions in the Notch signaling pathway and is altered through glycosylation by Lunatic fringe (LFNG). Feedback loop models have been proposed to explain interactions of Notch proteins in both the clock and patterning activities. In the clock, Hairy and enhancer of split 7 (HES7) and LFNG, which are transcriptionally activated by nuclear NICD, regulate each other and Notch, resulting in cyclic pulses of active Notch (Dale et al. 2003)(Morimoto et al. 2005). Furthermore, in region II of the PSM the patterning of somites depends on a rostral/caudal patterning feedback loop of the Notch ligands Deltalike 1 (DLL1) and Deltalike3 (DLL3) and the transcription factor MESP2 (Fig. 6) (Takahashi et al. 2003).

V. Specific Roles of Notch Signaling Pathway Genes

Mutations affecting Notch family members disrupt both the clock function and somite patterning, leading to skeletal deformation and disease in model organisms and in humans

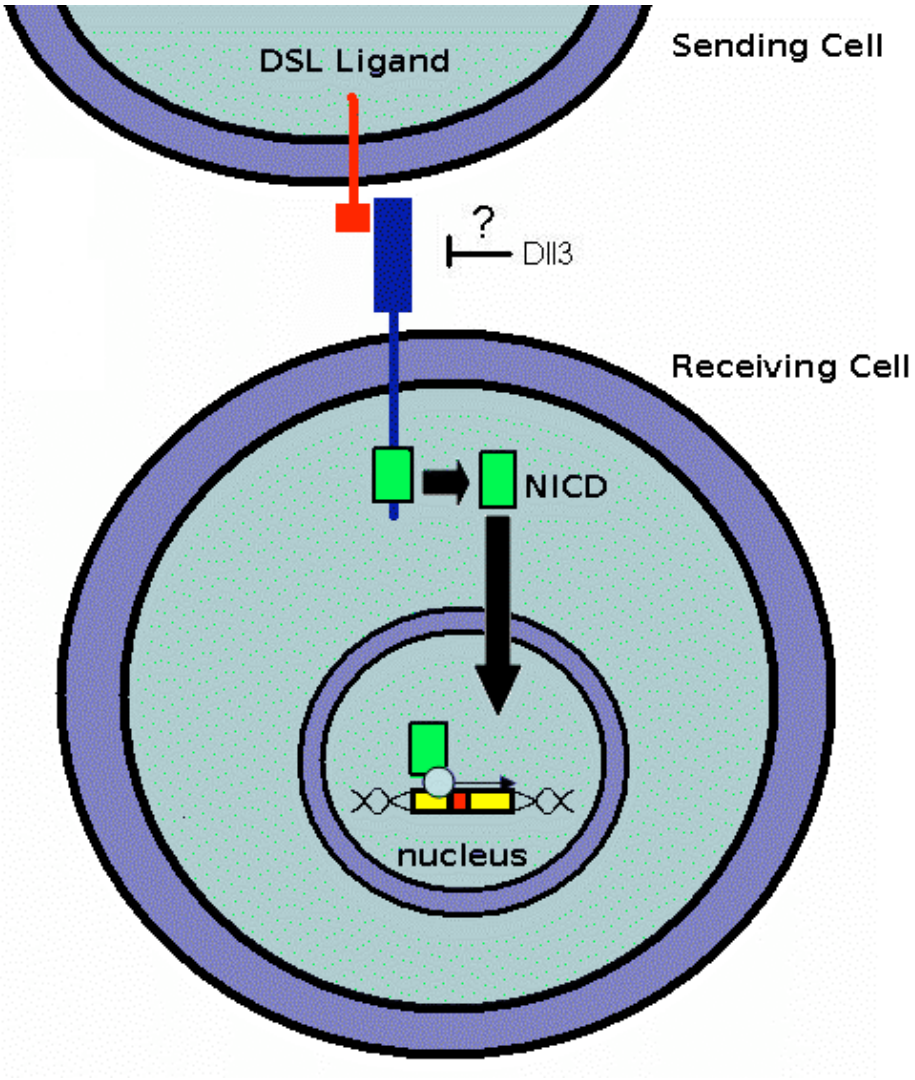


Figure 5. Simplified Notch Signaling Pathway. Notch is transactivated by interacting with a DSL ligand in another cell. Cleaved Notch, or NICD, translocates to the nucleus where it is a transcriptional co-activator for *HES* and *Fringe* genes as well as for *Nrarp* and other targets. The role of DLL3 in this pathway remains obscure.

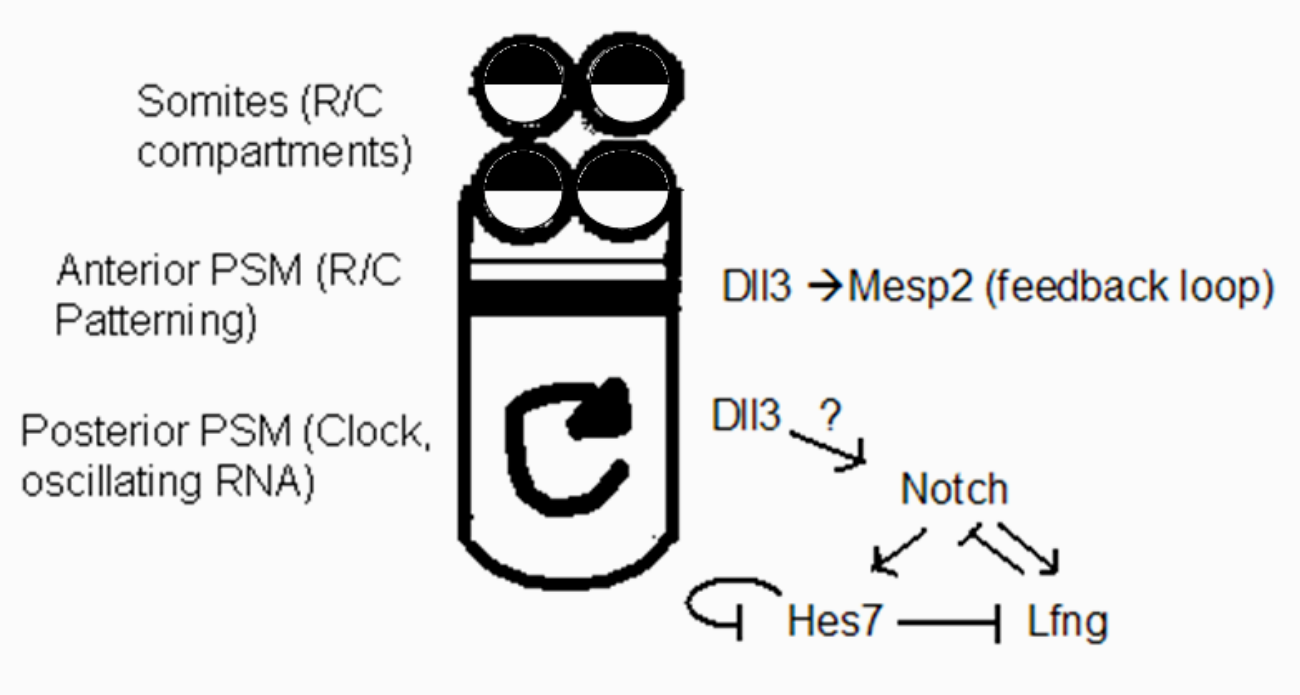


Figure 6. Regulation of Notch Signaling in the PSM. The segmentation clock in region I may be regulated by a negative feedback loop involving Notch, Hes7, Lfng and Dll3. The patterning of somites in region II involves a feedback loop, which includes the activities of Dll3, Dll1, and Mesp2. Refer to Figure 8 for more detail about the feedback loop involved in the patterning of somites.

(reviewed in Shifley et al. 2007). Loss of Lunatic fringe (*Lfng*), demonstrates how unperturbed Notch signaling is necessary for proper somitogenesis. *Lfng* null mice have malformed vertebrae, truncated tails, and fused vertebrae (reviewed in Shifley et al. 2007). Recently a mutation in LFNG was identified in human patients with spondylocostal dystostosis (SCD) (Sparrow et al. 2006) (reviewed in Shifley et al. 2007). LFNG functions by glycosylating and thereby modulating Notch (Maloney et al. 2000) (Sparrow et al. 2006). *Lfng* has roles in both region I, the segmentation clock, and region II, where somites are patterned, of the PSM. In *in situ* analysis of wild type mouse embryos, *Lfng* RNA oscillates in region I and has stable expression in region II (Fig. 6) (reviewed in Shifley et. al. 2007). Because Notch signaling pathway genes may be involved in either the clock or patterning or both, elucidating their roles in somitogenesis is challenging.

Understanding of the roles of members of the Notch signaling pathway is further complicated by cross-talk between the Notch pathway and the Wnt signaling pathway, which is also involved in clock activity during somitogenesis. For example, notch regulated ankyrin repeat protein (NRARP) oscillates in the posterior PSM, and it has separable roles in the Notch and Wnt pathways, suggesting that it may serve as a point of communication between the two pathways. NRARP has been shown to inhibit Notch by degrading NICD and to promote Wnt signaling, and it is a direct transcriptional target of the Notch pathway (reviewed in Shifley et al. 2007).

VI. Dll3's Role in Somitogenesis

Mutations in Deltalike 3 (DLL3), another protein involved in the Notch pathway, further demonstrate that unperturbed Notch signaling is necessary for proper somitogenesis. Loss of

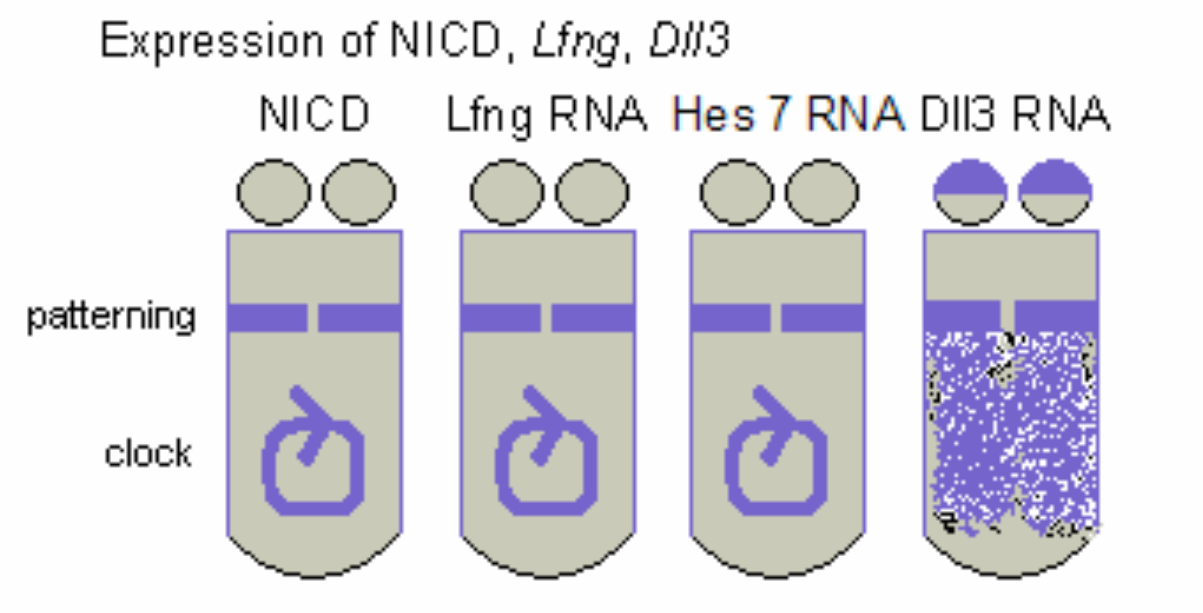


Figure 6. Wild Type expression of oscillating Notch pathway genes. Schematics that represent expression using in situ analysis are shown. NICD, *Lfng* RNA, and *Hes7* RNA levels oscillate in the posterior PSM where the clock is active and are stabilized in the anterior PSM where somites are patterned. *Dll3* RNA expression is ubiquitous in the clock region and stabilized in the anterior region.

the *DLL3* gene is responsible for Type I SCD. A mutation in DLL3 is found in 25% of people with SCD, who are characterized by rounded vertebrae (also known as a "pebble beach" sign), severe shortening of the trunk, and protrusion of the abdomen. Humans with SCD have vertebral and rib disorganization, caused by disrupted somitogenesis (Sparrow et al. 2006) (Kusumi et al. 1998). In mice, loss of *Dll3* results in the pudgy (pu) phenotype, arising from a disordered skeleton modeling the human disease (Kusumi et al. 1998) (Dunwoodie et al. 2001). Interestingly, the pudgy phenotype closely resembles that seen in *Lfng* null mice, suggesting that these two genes may have overlapping roles during Notch signaling (Fig. 7). The phenotypes in humans and mice resulting from a loss of Dll3 indicate that a disruption of somitogenesis has occurred and that DLL3 plays a critical role in the Notch signaling pathway. However, the roles of *Dll3* in Notch's clock and patterning activity are not fully understood.

VIII. Dll3's clock vs. patterning activity

Numerous studies have examined the role(s) of *Dll3* during somitogenesis. *In situ* RNA analysis of mouse embryos with a loss of *Dll3* identify different effects on the oscillatory RNA expression of the Notch signaling pathway members than those that are observed in wild type and *Lfng* null mice. For example, in *in situ* hybridization assays of wild type mouse PSM, *Lfng* and *Hes7* RNA expression patterns are oscillatory in the posterior PSM (clock, region I), showing multiple distinct phases in this region (Fig. 6). *In situ* hybridization assays of pudgy mice ($Dll3^{pu/pu}$) show that early in development, expression of *Lfng* RNA is similar to wild type, however *Lfng* cycling is lost later on in development. In contrast, expression of *Hes 7* RNA demonstrates maintained dynamic expression even in the absence of *Dll3* (Kusumi et al. 2004). On the other hand, in *Lfng* null embryos, cyclic transcription of *Lfng* is lost in region I but

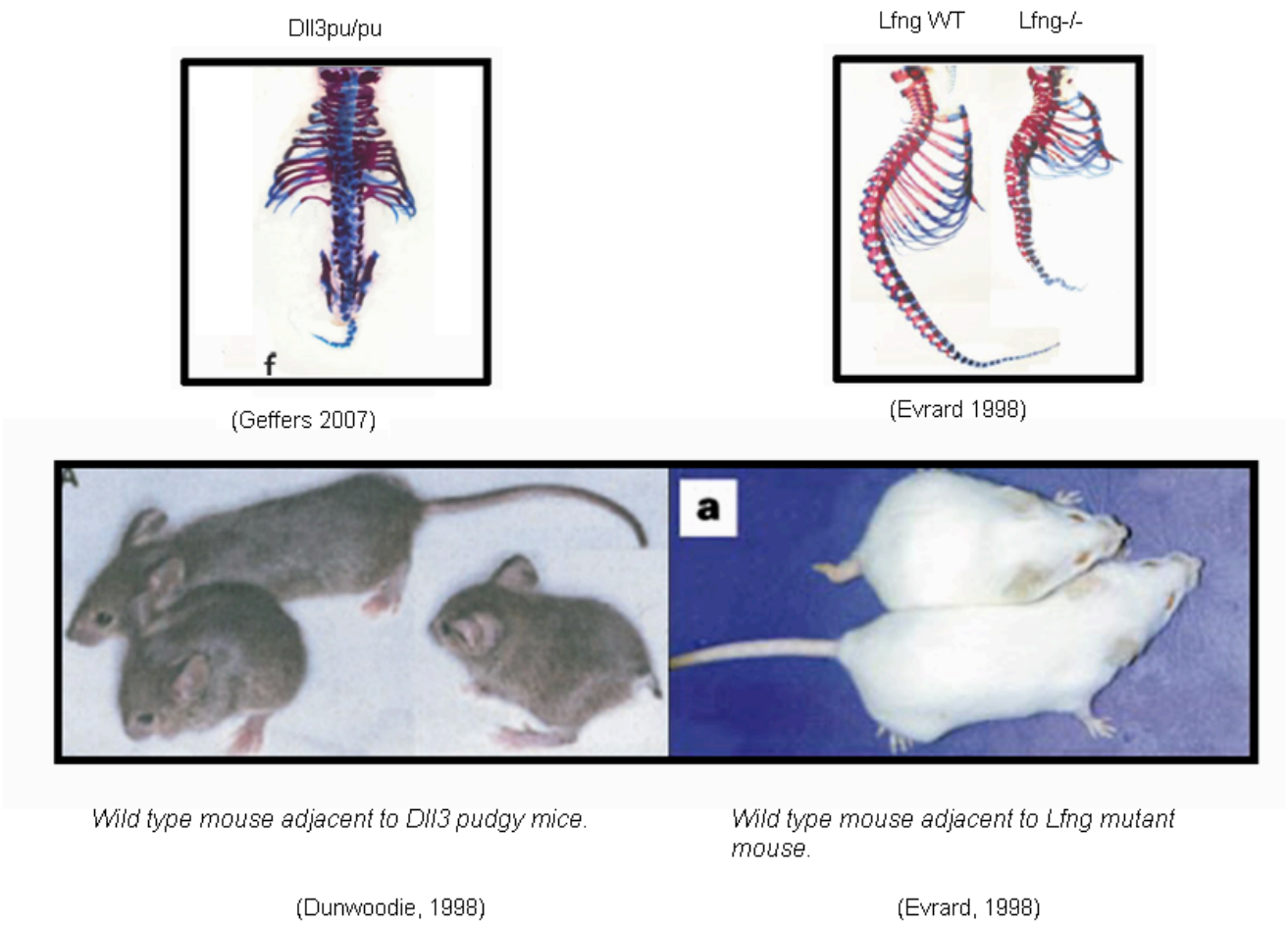


Figure 7. Dll3pu/pu and Lfng-/- mice. The pudgy mouse phenotype of a disordered skeleton and body and tail truncation is similar to that seen in Lunatic fringe nulls.

transcription is maintained in region II (unpublished data). *Hes 7* is expressed ubiquitously early and cyclically later in mice with a deletion of *Lfng* (Shifley et al. 2008). Evidently, *Dll3* affects the clock function of the Notch pathway, but its role is unclear because the *in situ* findings of *Dll3* null embryos differ from that of *Lfng*.

Dll3's role is better understood in patterning. *Mesp2* is considered a stage specific gene because it is expressed as a single band in region II (patterning activity) and not region I (clock activity) of the PSM. It is involved in segmental border and rostral/caudal (RC) demarcation. *Dll1* and *Dll3* are also implicated in patterning activities in the PSM because losses of these genes result in a disruption of rostral/caudal polarity of somites. It has been suggested MESP2 might be a mediator of DLL1 and DLL3's patterning activities, so MESP2 was tested to see if it had a more direct role in patterning. (Takahashi et al. 2003)

In order to determine the feedback loops regulating *Dll1* and *Mesp2* during stripe formation, *Dll1* expression in *Dll1* null mice was compared to previous findings of *Mesp2* expression in *Dll1* null mice and *Dll1* expression in *Mesp2* null mice (Takahashi et. al., 2000) (Takahashi 2003). The findings from these studies suggest that *Dll1* activates *Mesp2* and itself. Also, *Mesp2* inhibits *Dll1*, but not itself. In situ analysis of *Dll1* null, *Mesp2* null, and *Dll1/Mesp2* double null embryos for the patterning genes, *Cer1* and *Uncx4.1*, suggest that *Mesp2* has a more direct role in determining R/C polarity than does *Dll1*. For instance, in *in situ* analysis of *Mesp2* null embryos, there is an expansion of *Dll1* expression but a loss of *Cer1* expression compared to expression patterns seen in wild type embryos. Because there is also a loss of *Cer1* expression in *Dll1* null embryos, *Mesp2* appears to act more directly than *Dll1* on *Cer1*. The results of similar studies examining the relationship between *Dll3* and *Mesp2* suggest

that both *Dll3* and *Mesp2* are needed for their mutual expression. Also, the data suggest that during rostral/caudal patterning, *Mesp2* is located downstream of *Dll3*. For example, *Dll3* is required for proper localization of *Mesp2* during R/C patterning. Taken together, this data suggests a feedback loop involving *Dll1*, *Dll3* and *Mesp2* for the rostrocaudal patterning of somites. After segregation of cells into rostral/caudal compartments, *Dll3* and *Mesp2* suppress *Dll1* and *Uncx4.1* in the rostral region while *Dll1* induces itself and *Uncx4.1* in the caudal half (Fig. 8) (Takahashi et al. 2003).

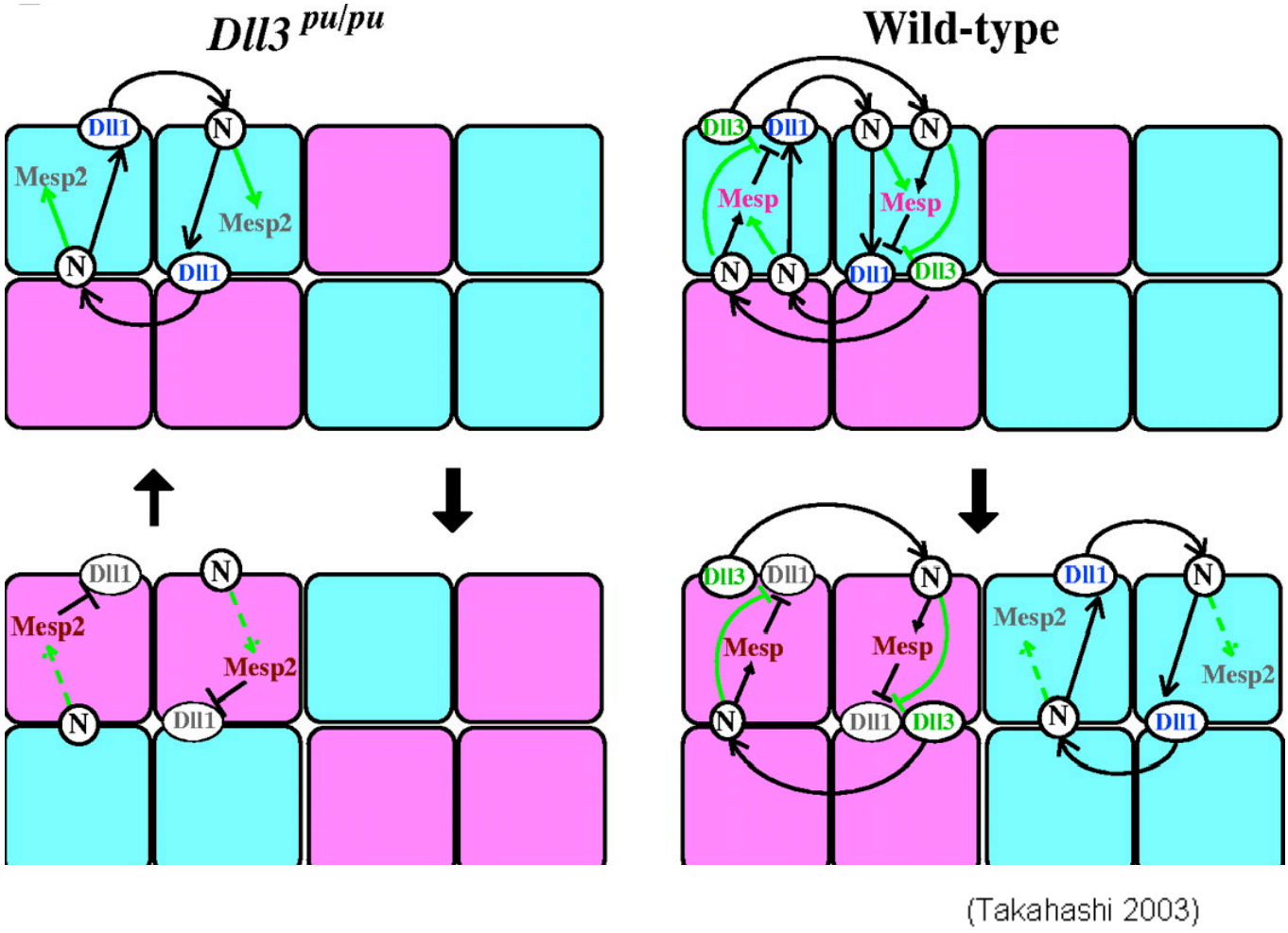


Figure 8. Roles of Dll3, Dll1, and Mesp2 in R/C somite patterning. *Dll1*, *Dll3*, and *Mesp2* form a feedback loop responsible for the Rostral/Caudal patterning in the PSM.

IX. Dll3's Role as an Activator or Inhibitor of Notch

Up to this point, data implicating *Dll3*'s role in the Notch signaling pathway is contradictory. *Dll3* and *Dll1* are the only DSL ligands expressed in the PSM whose mutations disrupt somitogenesis (Dunwoodie et al. 2002) (Zhang et al. 2002). *Dll1* is a known activator of Notch, but the role of *Dll3* as an inhibitor or activator of Notch is less well understood. As discussed earlier, DSL ligands are required for the trans activation of Notch signaling and *Dll3* was originally described as a Notch ligand (Dunwoodie et al. 2002) (Kusumi et al. 1998). In some studies, *Dll3* as been implicated as a DSL ligand that activates Notch, but there has also been data used as evidence against this theory that in fact suggests that *Dll3* may have a role as a Notch inhibitor. Originally, analyses in *Xenopus* embryos showed the inhibition of primary neuron formation by DLL3. *Dll3* and *lacZ* RNA were co-injected into a *Xenopus* blastula at the 2-to 4- cell stage, and embryos were cultured to the neural plate stage. *In situ* analysis showed that *N-tubulin* expression decreased in the neural plate. As *N-tubulin* expression is inhibited by active Notch, this suggested that the DLL3 ligand was able to bind and activate Notch in this assay (Dunwoodie et al. 1997). However, in tissue culture analyses, researchers found that DLL3 is unable to activate Notch signaling in trans. Instead, researchers found that Dll3 is able to cell autonomously inhibit Notch signaling, acting as an antagonist to DLL1 activation (Ladi et al. 2005). One should note that the differences in experimental models and methods used between the two studies may play a role in the different conclusions made about whether *Dll3* is an activator or inhibitor of Notch, especially since they represented in vitro modeling using over-expression techniques. A consistent and systematic in vivo study is needed to clarify the specific roles of this Notch family member during somitogenesis.

To clarify conflicting evidence about Dll3's interaction with Notch, *in vivo* experiments were performed to see if *Dll3* and *Dll1* are antagonists during development. After targeted insertion of *Dll3* into the *Dll1* locus, mice were created with varying ratios of *Dll3* to *Dll1* and no phenotypic evidence of functional antagonism was observed. Also tissue culture data showed that DLL3 and DLL1 have different cellular localizations; DLL3 has a more intracellular localization than DLL1, suggesting further that they are less likely to be direct antagonists. These results suggest that *Dll1* and *Dll3* do not antagonize each other during Notch signaling, but they do not specifically answer whether *Dll3* is an activator or inhibitor of Notch (Geffers et al. 2007).

XI. Phenotypes: Dosage effects of Notch signaling genes

Lfng and *Dll3* are co-expressed in cells during somitogenesis; however, little is known about their interaction during Notch signaling. Recent investigation indicates that mutations affecting distinct Notch pathway members can give rise to unique phenotypes. Mice with double heterozygous mutations in *Dll3* and *Notch* (*Dll3*+/-; *N*+/-) exhibit an intermediate skeletal phenotype, unique from the phenotype exhibited by a homozygous mutation in either gene. These mice have vertebral fusions, mid-line fusion defects, and anomalously enlarged rib regions. This research suggests that skeletal phenotypes found in humans or model organisms may result from synergistic effects of two or more genes, not just from mutant or haploinsufficiency of one gene, in the Notch signaling pathway (Loomes et al. 2007). Because loss of a single *Dll3* allele appears to be able to act in combination with heterozygous mutant alleles of other Notch pathway genes to affect defects in somitogenesis, this double mutation technique can be used to analyzed *Dll3*'s interaction with *Lfng*.

XII. Conclusion and Specific Aims

The severity of the mouse pudgy phenotype and the human disease SCD reflects the importance of Dll3 in the Notch signaling pathway and for proper somitogenesis. However, there is still very little known and there is inconclusive evidence about *Dll3*'s specific role(s) in the pathway. Because mice with deletions in *Dll3* have both a perturbed clock function and abnormal somite patterning, with just this evidence, it is impossible to determine which aspects of the pudgy phenotype are due to problems with the clock, problems with the somite patterning, or both. Furthermore, elucidating *Dll3*'s role as an activator or inhibitor of Notch requires more *in vivo* experiment data. Finally, it is important to understand whether *Dll3* acts independently or is modified by other Notch family members in affecting unique skeletal phenotypes. Therefore, my project will be addressing the following questions: (1) Does Dll3 activate or inhibit Notch signaling in the PSM? (2) Does Dll3 play critical roles in the clock or somite patterning or both? (3) Do unique skeletal phenotypes arise from modifier effects between *Dll3* and *Lfng*, Notch family members?

Because *Dll3* pudgy mice share similar skeletal phenotypes with *Lfng* null mice, I am performing experiments that were used to determine whether *Lfng* is an activator or inhibitor of Notch on mouse embryos. By analysis of NICD levels (which reflect Notch activity) and NRARP RNA (which reflects expression of a Notch target gene) in pudgy mouse embryos, we hope to determine how the loss of Dll3 affects activation of Notch. In order to address whether the pudgy phenotype results from a loss of Dll3 in the clock or patterning regions of the PSM during somitogenesis, we are examining a skeletal phenotypic rescue in pudgy mice that only express *Dll3* in the anterior PSM. Finally, in order to analyze modifier effects of Notch family

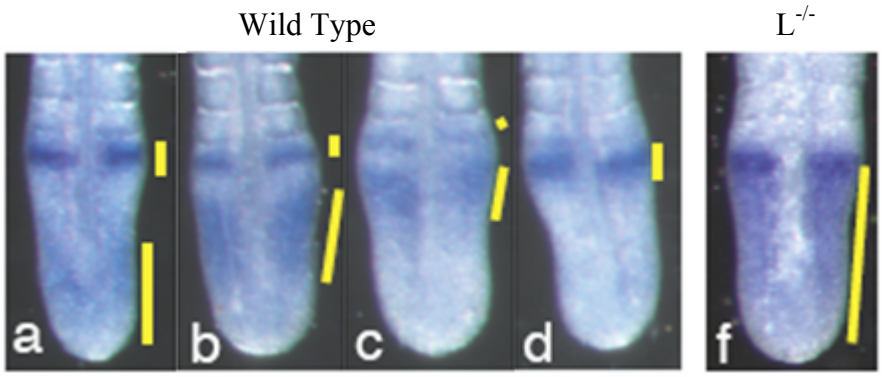
members, we are looking for intermediate skeletal phenotypes in double heterozygous *Dll3-Lfng* mouse embryos.

By gaining a better understanding of the specific roles of *Dll3* in a complicated signaling pathway, we will better understand the process of somitogenesis and the mechanisms that regulate it.

II. Results

Dll3 expression is required for Notch 1 activation in Region I of the PSM

The Notch receptor becomes activated by cleavage, and the activity of NICD in the nucleus co-activates the transcription of the proteins involved in the Notch signaling pathway. In the somitogenesis clock, Notch experiences periodic pulses of activation and inhibition, coinciding with the reiterated pattern of pre-somite patterning and somite budding. This cyclic activity can be observed by using whole mount immunohistochemistry with an antibody specific for the cleaved, activated form of Notch 1 (NICD). Because loss of *Lfng* results in disruption of both the somitogenesis clock and patterning, NICD activity was analyzed in *Lfng* null embryos to understand the activation or inhibition of Notch that results in periodicity in the posterior PSM. In whole mount analysis of NICD expression in *Lfng* null embryos, ubiquitous expression of NICD in the clock was observed (Fig. 9) (Shifley et al. 2008). These experiments may suggest that *Lfng* inhibits Notch signaling in the clock (Morimoto et al. 2005)(reviewed in Shifley et al. 2007). *Dll3*, an unusual ligand, also has important roles in the Notch pathway during segmentation but studies have not yet clarified whether it acts as an activator or inhibitor of Notch, with recent evidence suggesting that it is an inhibitor (Ladi 2005). In order to test the affect of *Dll3* on oscillatory Notch activation, we performed whole mount analysis of NICD



(Shifley 2008)

Figure 9. Whole mount analysis of NICD in the PSM of Lfng$^{-/-}$ mouse embryos (10.5 d.p.c.). A-D NICD expression oscillates in region I of wild type embryos. **F** NICD expression is ubiquitous in region I in the *Lfng* null embryos.

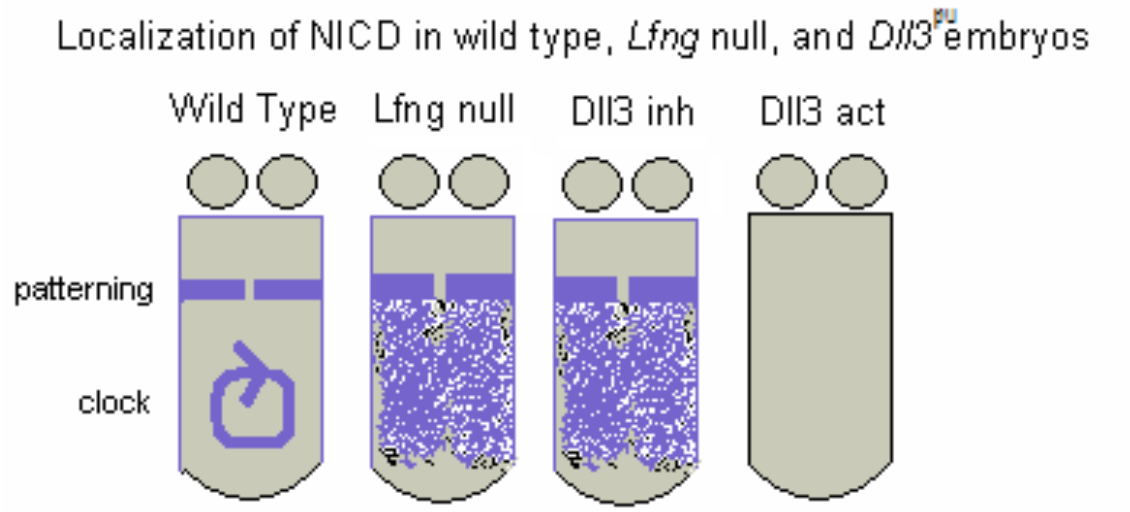


Figure 10. Activation of NICD in wild type and gene knock out embryos. Schematics that represent expression using Whole Mount Immunohistochemistry are shown. In wild type embryos, NICD levels oscillate in the posterior PSM where **t**he clock is active and are stabilized in the posterior PSM where the somites are patterned. In *Lfng* null embryos, NICD expression is ubiquitous in the posterior clock region and the expression levels are stabilized in the anterior PSM. Analysis of NICD in *Dll3*$^{pu/pu}$ embryos to determine the role of Dll3 as an activator or inhibitor of Notch. Expected results: If *Dll3* inhibits *Notch*, then we may observe ubiquitous expression of NICD in the clock region. If *Dll3* activates *Notch*, then we may observe loss of NICD in the PSM.

expression in pudgy embryos in order to assess the activation of Notch. If ubiquitous expression of NICD in the posterior PSM is observed in pudgy embryos as in *Lfng* null embryos, then this might suggest that *Dll3* acts as a Notch inhibitor in the clock (Fig. 10). In addition, an up-regulation in levels of NICD would suggest that a loss of *Dll3* results in a loss of inhibition of Notch activation.

Despite the similarities between the *Lfng*$^{-/-}$ and *Dll3*$^{pu/pu}$ phenotypes, NICD localization in pudgy embryos does not resemble that in *Lfng* null embryos. As expected, four phases of NICD expression were visualized in whole mounts of wild type embryos, indicating oscillatory activity of Notch in the posterior PSM and serving as a positive control for the whole mounts of the pudgy embryos that were assayed simultaneously. In the PSM of the pudgy embryos, no NICD expression was observed in region I, suggesting that in the absence of *Dll3*, Notch1 is not activated in the clock (n=10) (Fig. 11). However, NICD expression appears in region II as a single stripe, suggesting that *Dll3* is not required for Notch activation during R/C somite patterning. Further experiments will be required to determine whether NICD is properly regulated or localized in region II of *Dll3*$^{pu/pu}$.

As an alternate method to examine Notch I activity in the *Dll3* null embryos, we examined the expression of *Nrarp*, a direct transcriptional target of the Notch pathway. As in the NICD experiment, there was a loss of *Nrarp* expression in region I and there was a single stripe of expression in region II of the pudgy embryos (n=3) (Fig. 12). More embryos must be assayed to show significant results. In both the NICD and NRARP whole mounts, levels of NICD and NRARP expression in pudgy embryos did not increase compared to wild type embryos that were placed in detection solution for the same amount of time. These findings suggest that inhibition of Notch is not lost in the anterior PSM in pudgy embryos

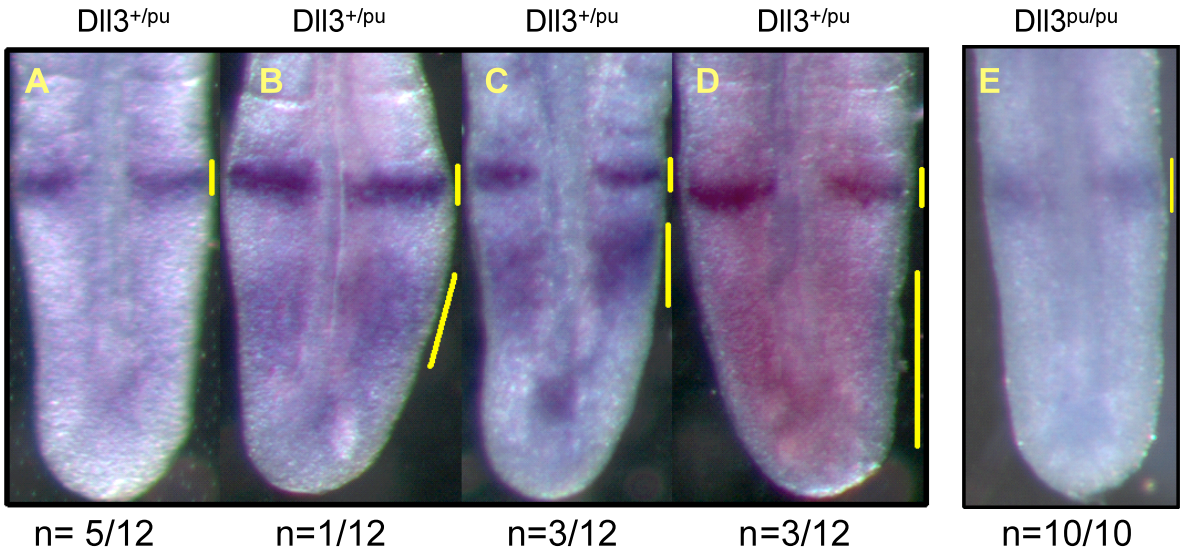


Figure 11. Whole mount analysis of NICD in the PSM of Dll3$^{pu/pu}$ mouse embryos (10.5 d.p.c.). A-D represent the PSM of wild type embryos exhibiting four phases of oscillating NICD expression. **E** represents the PSM of Dll3pu/pu embryos. Expression of NICD is observed only in the anterior PSM of pudgy embryos (10 embryos assayed). No NICD is observed in Region I. Also, the amount of NICD is not up-regulated compared to **C** and **D** (same amount of time in detection solution). *Dll3* is necessary for Notch activation in the posterior PSM but not in the anterior PSM. These findings are dissimilar to results of these studies in *Lfng* knockouts.

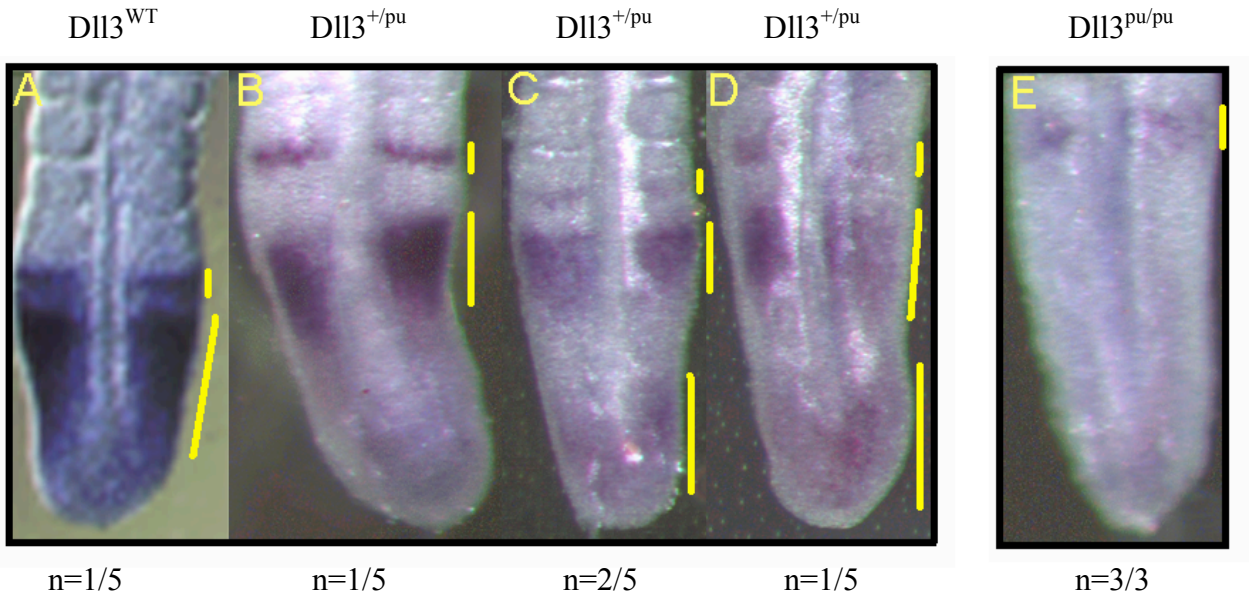


Figure 12. Whole mount In situ analysis of NRARP in the PSM of Dll3pu/pu mice (10.5 d.p.c.). A-D represent the PSM of wild type embryos expressing four phases of oscillating NRARP expression. **E** represents the PSM of Dll3pu/pu embryos. As in the NICD whole mounts, NRARP expression is not observed in the posterior PSM of pudgy embryos (3 pudgy embryos tested). Also, the level of NRARP expression in the anterior PSM is reduced compared to the wild type. *Dll3* has a role in the activation of Notch signaling in the posterior PSM but not in the anterior PSM. These results are inconsistent with the studies of NRARP in *Lfng* null mice.

The loss of oscillatory NICD, active Notch, and Nrarp, a direct transcriptional target of Notch, in pudgy embryos suggest that *Dll3* is required for Notch activation in the clock. Because there is partial rescue of NICD and Nrarp expression in the anterior PSM, *Dll3* activation is not required for R/C patterning.

Studies are in progress to dissect the roles of Dll3 in the clock and patterning activities during segmentation

Loss of *Dll3* could disrupt both the clock and patterning activities of the Notch signaling pathway. Disturbance of both the clock and patterning activities is also seen in *Lfng* null mice. In mice homozygous for the $Lfng^{\Delta FCE1}$ mutant alelle, expression of wild type *Lfng* is only perturbed in the clock, and we find that these mice have segmentation defects in the thoracic and lumbar vertebrae but not in the sacral and tail regions. These findings suggest that the periodic nature of Lfng in the clock is necessary for Notch signaling during anterior skeletal development but not during posterior skeletal development (Shifley et al. 2008). Further, these experiments demonstrated that *Lfng* plays distinct, separable roles in the clock vs. patterning. Because *Lfng* and *Dll3* nulls exhibit similar skeletal phenotypes, a similar analysis of pudgy mice only expressing *Dll3* in the anterior PSM could be done in order to better understand its clock vs. patterning activities.

By driving expression of Dll3 only in the anterior PSM, we hope to specifically rescue aspects of the *Dll3* phenotype that relate to R/C patterning. A transgene (pSEC169) was designed utilizing the *Mesp2* promoter to drive *Dll3* expression specifically in the anterior PSM (Fig. 13) (Haraguchi et al. 2001). Founder mice expressing the transgene ($Dll3^{+/+}$, tg+) would be bred with heterozygous or homozygous pudgy mice ($Dll3^{+/pu}$; tg- or

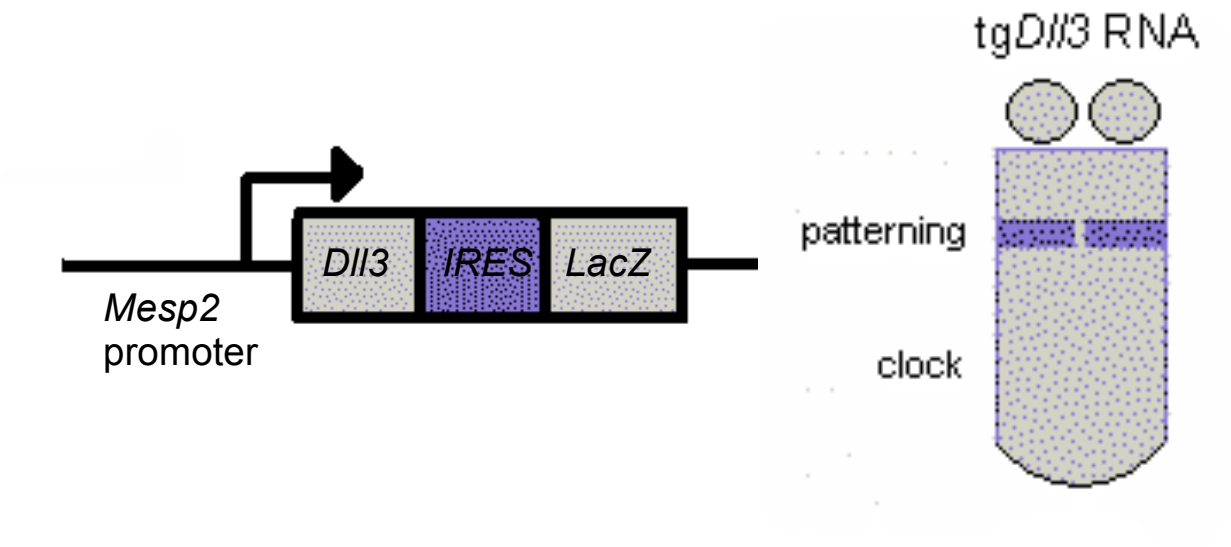


Figure 13. Creating a transgene that localizes *Dll3* in the anterior PSM. Schematic representation of a transgene (pSEC169) driving *Dll3* expression in the anterior PSM and its expected expression pattern.

Dll3$^{pu/pu}$; tg-) in order to analyze offspring that were homozygous for the pudgy allele but which express *Dll3* in the anterior PSM from the transgene (*Dll3*$^{pu/pu}$; tg+). These offspring would only express *Dll3* in the anterior PSM but not in the posterior PSM. These would then be examined by the skeletal preparations protocol at 17.5 d.p.c. and analyzed for a rescued phenotype. If the mutant embryos had a wild type skeleton, then these results would suggest that *Dll3* is more important in the patterning activity of the Notch signaling pathway than in the clock activity. If the mutant embryos had a pudgy skeletal phenoytpe, then *Dll3* would apparently be more important in the clock activity than patterning because *Dll3*'s exogenous expression only in the anterior PSM did not rescue the wild type phenotype in pudgy embryos. Furthermore, an intermediate phenotype between the pudgy and wild type skeleton would suggest incomplete rescue and a more complicated role for *Dll3* in the clock or patterning.

Despite successful injection of pSEC169, resulting in 5 male and 2 female founders that transmitted the transgene to their offspring, we do not observe expression of the exogenous *Dll3* in the anterior region of the PSM (Fig. 14). Assays for expression of the transgene in offspring embryos was performed at 10.5 d.p.c. using whole mount RNA hybridization and the *Lac Z* Staining of embryos protocol.

These results may indicate that the previously published *Mesp2* promoter does not robustly drive expression in the anterior PSM as expected.

Embryos heterozygous for mutations at both the *Dll3* and *Lfng* loci do not exhibit any skeletal phenotypes

Complete loss of either *Dll3* or *Lfng* results in a shortened body axis and a truncated tail and very similar skeletal phenotypes. One possible assumption could be that these two *Notch* family members' contributions to normal somitogenesis and skeletal development

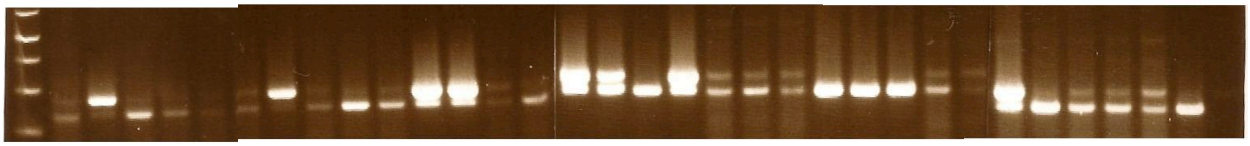
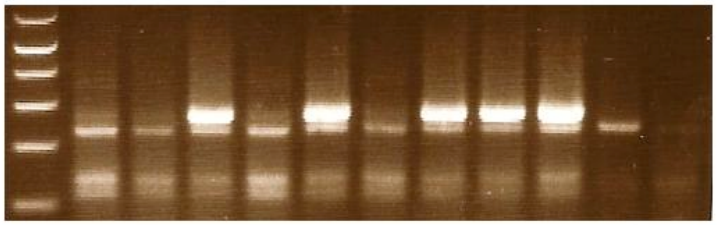
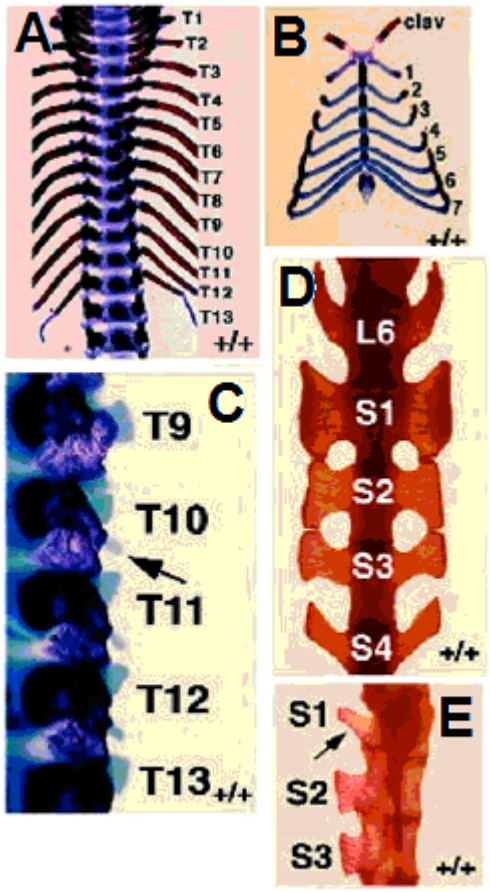


Figure 14. Wild type founders expressing pSEC169. The control is represented by the 200 bp (lower) band and the transgene is represented by the 257 bp (upper) band. After injection of pSEC169, 5 male and 2 female mice were found that had incorporated the transgene. These founders were bred to assay the transmission and expression of pSEC169 in their offspring.



Offspring of founders who express pSEC169. The control is represented by the 200 bp (lower) band and the transgene is represented by the 257 bp (upper) band. The embryos who were positive for the transgene were assayed with Whole Mount In Situ analysis and *lacZ* staining to probe for expression of pSEC169. Further analysis must be performed to identify embryos expressing the transgene.

might be co-dependent. Therefore, whether these genes have overlapping activities during skeletal development was analyzed. Unique skeletal phenotypes were found in mice that were double heterozygous mutant in two *Notch* family proteins, *Notch 1* and *Dll3*, suggesting the possibility that somitogenesis can be perturbed by dosage effects of multiple rather than any single *Notch* gene (Loomes et al. 2007). To test the possibility that *Dll3* and *Lfng* show similar evidence of synergistic effects, the skeletons of 17.5 d.p.c. embryos that were heterozygous mutant and homozygous null for both *Dll3* and *Lfng* (*Dll3*+/pu; *Lfng*+/- and *Dll3*pu/pu; *Lfng*-/-) were analyzed using the skeletal preparations protocol (Fig. 15). The double heterozygous mutants (n=14) were identical to wild type and single heterozygous mutants (Fig. 16) (Fig. 16). Experimentation is in progress to analyze the double null mouse embryos.



(Barna, 2000)

Figure 15. The wild type mouse skeleton. L+/-; D+/pu mouse skeletons were examined for the following wild type characteristics: (a) 13 thoracic vertebrae, (b) 7 ribs attached to the sternum, (c) 6 lumbar and 4 sacral vertebrae, (d) fused processes on S1-S3.

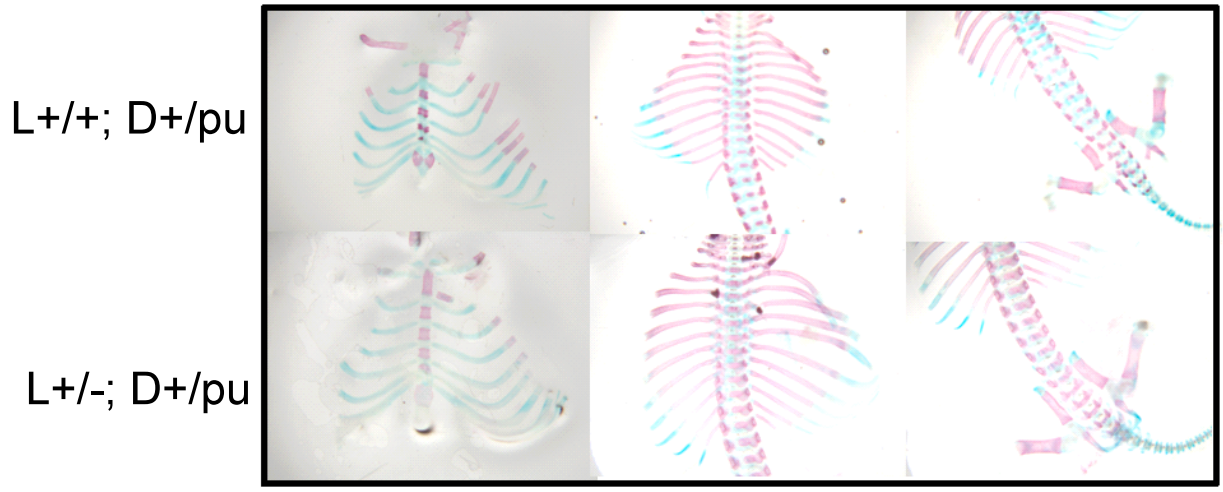


Figure 16. Skeletal phenotypes of wild type and L+/-; D+/pu mouse embryos (17.5 d.p.c). The skeletal phenotypes of 14 double heterozygous embryos were compared to wild type mouse skeletons. There were no observable differences between the mutant and wild type phenotypes.

III. Discussion

The original hypotheses of Dunwoodie (*Dll3* is a Notch activator) and Ladi (*Dll3* is a Notch inhibitor) about the role of *Dll3* have been clarified by recent in vivo findings in the Geffers study and the whole mount and skeletal prep data presented here that support that *Dll3* is an activator of *Notch* signaling (Geffers et al. 2007). The analyses presented by the Dunwoodie and Ladi experiments over-expressed *Dll3* in the experimental models, which could create an artificial environment and perhaps account for the discrepancies in the results of their studies (Dunwoodie et al. 1997) (Ladi et al. 2005).

Although *in vitro* data was useful in the early analysis of the unusual ligand *Dll3*, there is currently more background information available that can help interpret *in vivo* findings. *In vivo* experimental methods used to interpret *Lfng* were used to analyze *Dll3* because these genes have been implicated to have similar roles during Notch signaling. The finding that NICD expression is ubiquitous in *Lfng* null embryos but that NICD and NRARP expressions are lost in the posterior PSM of *Dll3* pudgy embryos suggests that *Dll3* is not a Notch inhibitor like *Lfng*. Furthermore, because there is no evidence of up-regulation of NICD or NRARP in the pudgy embryos, a loss of *Dll3* does not allow for increased expression levels of activated Notch signaling in the anterior PSM. Therefore, the lost activity in the clock and the lower levels of NICD or NRARP expression in the anterior PSM suggest that *Dll3* is not an inhibitor, but an activator of *Notch*.

Furthermore, a similar experiment of active Notch expression in mice lacking *Dll3* (pudgy or pu) by assaying NICD was recently included in a publication. The researchers likewise found that in pudgy embryos, NICD expression is lost in the posterior PSM. Also, there

was no up-regulation of levels of expression of NICD in the *Dll3*^{pu} embryos, further supporting that *Dll3* is not an inhibitor of Notch (Geffers et al. 2007).

When *Dll3* was implicated as an inhibitor of Notch, *Dll3* was suspected to be an antagonist of *Dll1* (Notch activator), another DSL ligand expressed in both the clock and patterning regions of the PSM. However, recent data suggests that they are not antagonists and that, in fact, they may act in different parts of the cell during Notch signaling (Geffers 2007). Therefore, *Dll1* has been implicated in trans-activation of the Notch receptor and *Dll3* has been suggested to somehow act in the Golgi during Notch signaling. Because *Lfng* appears to inhibit the Notch receptor in the Golgi and because *Dll3* and *Lfng* are co-expressed in cells, it is possible that *Dll3* is involved as an antagonist of *Lfng*.

Because both *Dll3* and *Lfng* null mice have similar phenotypes and the genes both localize in the Golgi, *Dll3* may also function in modulation of the Notch receptor. *Dll3* has been implicated in the negative feedback loop responsible for periodic activation of Notch signaling in the clock (Ladi 2005). However, data suggesting that *Dll3* and *Lfng* are both involved in Notch modulation in the Golgi does not necessarily suggest that they are either co-dependent or antagonists. *Dll3*'s interaction with Notch has also been shown to be independent of *Lfng* glycosylation (Ladi 2005). Also, DLL3's localization in the Golgi might only be important at different time points (d.p.c) or only during the clock or patterning activities of Notch signaling. Furthermore, DLL3 might instead be acting indirectly through other genes instead of on the Notch receptor itself.

The theory that *Dll3* activates Notch signaling might be supported by implications of the skeletal phenotypes of the double heterozygous mutant embryos for *Dll3* and *Lfng* (D+/pu; L+/-). Loss of one allele of each gene does not result in an intermediate phenotype

as seen in mice heterozygous for *Dll3* and the Notch receptor (D+/pu; N+/-). This new phenotype may arise because *Dll3* and *Notch1* have a synergistic relationship, and a phenotype is not seen in the D+/pu; L+/- mice because *Dll3* and *Lfng* counteract each other in *Notch* signaling. If we assume that *Dll3* is an activator of *Notch*, then the combinatorial effects of a loss of one allele responsible for the activation of Notch signaling (*Dll3*'s role) and the loss of one allele representing active notch (Notch receptor) can together account for a disruption of somitogenesis. However, in the D+/pu; L+/- embryos, we do not seen combinatorial effects, perhaps because the genes have antagonistic roles in Notch signaling: *Dll3* activates the Notch receptor and *Lfng* inhibits the activation of the Notch receptor.

Although the similar phenotypes of *Dll3* and *Lfng* nulls suggest redundant roles during somitogenesis, the loss in *Dll3* and *Lfng* result in two subtly different types of human disease, SCD1 and SCD3 respectively (Sparrow 2006). These findings may suggest that *Dll3* and *Lfng* have different roles in somitogenesis at least in humans, necessitating further study of these differences in order to better understand human embryo somitogenesis and skeletal development.

Whether *Dll3* and *Lfng* act antagonistically in the segmentation clock needs to be addressed with further research. Recent investigations suggest that oscillatory *Lfng* is important for anterior skeletal formation and not tail development (Shifley et al. 2008). Therefore, other members of the Notch signaling pathway may have different roles at different times during somitogenesis. For instance, *Dll3* may act as an activator of Notch in the Golgi and *Lfng* may act as an inhibitor in the Golgi, but their activities may not overlap in the Golgi because they take place at different times in embryo development.

One possible study that could be performed in order to determine whether *Dll3* is more important in the clock during early or late somitogenesis would be to expand on the *in situ* analyses of *Hes7* in pudgy embryos at 9.5 d.p.c (Kusumi 2004). *Hes 7* is a transcriptional target of *Notch*, so expression of *Hes7* indicates active Notch signaling. At 9.5 d.p.c., *Hes7* expression is dynamic in pudgy embryos. In situ analysis of embryos at 8.5 d.p.c (before tail bud formation, anterior skeletal development) and 10.5 d.p.c (tail development) would clarify whether loss of *Dll3* disrupts Notch signaling in the segmentation clock during early or late somitogenesis. Additionally, the whole mount analyses of NRARP and NICD should be performed on 8.5 and 9.5 d.p.c. pudgy embryos to determine whether DLL3 is required in both early and later somitogenesis.

Because *Dll3* may have multiple roles during somitogenesis, fully understanding the acitvities of *Dll3* in *Notch* signaling will require more analysis, controlling for location of *Dll3* expression in the PSM and time d.p.c. Because of the possible antagonistic interaction between *Dll3* and *Lfng* suggested by these studies and other research, more investigation of their combinatorial effects on somitogenesis should contribute to a better understanding of *Dll3*'s roles in the Notch pathway. Also, a better understanding of the *Dll3-Lfng* interaction could advance explanations for somitogenesis defects and disease in mice and humans.

IV. Materials and Methods

Genotyping

Tail Preparations (Salt Out Protocol): First, 600 ul of TENS and 6 ul of ProteinaseK were added to 1 cm of mouse tail or embryo skin (17.5 d.p.c. for skeletal preparations) and incubated overnight at 55° C. The samples were vigorously shaken after adding 166.73 ul of

saturated NaCl. After centrifuging at top speed for 10 minutes, 600 ul of 95% EtOH was added to the supernatant in a fresh tube. After centrifuging, the pellet was washed in approximately 1 mL of 70% EtOH. The pellet was re-suspended in 100 ul of 10mM Tris pH 7.5.

Yolk Sac Preparations: Embryos were harvested using standard protocol and the yolk sacs were saved for genotyping. After adding 100 ul of 50 mM NaOH to the yolk sacs, the mixtures were placed on a boiling heat block for 15 minutes and then on ice for 5 minutes. Next, 25 ul of 1/3 M Tris pH 7.4 was added to the mixtures and after centrifuging, 90 ul of the supernatant was isolated from each sample.

PCR: *Dll3*^{pu} reactions were run using primers SC-355 or SC-320 and SC-356 for 35 cycles at 58° C and digested with HaeIII to distance the pudgy and wild type alleles. The mutant allele was amplified as 130 bp with the wild type at 103 bp. *Lfng* reactions were run using primers FNG-222, FNG-225, and PGK3 for 35 cycles at 55° C. The mutant allele was amplified as 200 bp with the wild type at 170 bp. pSEC169 reactions were run using primers SC-340, SC-342, and KT-53&54 for 35 cycles at 55° C. The transgene was amplified as 257 bp with the control at 200 bp.

Whole Mount Protocols

Acquisition and treatment of mouse embryos: Embryos were dissected in ice-cold Phosphate Buffered Saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS and 0.1% Tween-20 (PBT) at 4° C overnight. Embryos were dehydrated into methanol and stored at -20° C.

Antibody Staining for NICD: Embryos were rehydrated into PBT and then bleached by incubating in PBS containing 0.1% hydrogen peroxide, 1% Triton X-100 (TX-100) and 10%

fetal calf serum at 4° C overnight. The next day, embryos were washed and boiled in 10 mM NaCitrate (pH 6.0) with 0.1% Tween-20. They were incubated in a primary antibody (NICD)(Cell signaling technology) diluted 1:250 in PBS containing 1% TX-100 and 10% fetal calf serum for 5 days at 4° C. The embryos were then incubated in AP-tagged anti-rabbit IgG antibody diluted 1:500 in 100 mM Maleic Acid, 150 mM NaCL, and 0.1% Tween-20 to a pH of 7.5 using NaOH (MABT), 2% Boehringer Blocking Reagent and 20% heat-inactivated sheep serum overnight at 4° C. Post antibody washes were performed with a solution of MABT and 2mM Levamisole. The next day, embryos were incubated in 5 mLs of detection solution containing 100 mM NaCl, 100 mM Tris pH 9.5, 50 mM $MgCl_2$, and 1% Tween-20 (NTMT), 2mM Levamisole, 25.3 uL of NBT, and 13 uL of BCIP at room temperature covered in aluminum foil until desired staining was apparent. Then, the embryos were washed in PBT pH 5.5. Embryos were post-fixed in 4% PFA and 0.1% gluteraldehyde for 1 hour at room temperature. Embryos were then de-hydrated into methanol and re-hydrated into PBT. The embryos were then cleared by washing them in a 1:1 Glycerol:PBT solution for 1 hour and then stored in a 4:1 Glycerol:PBT solution.

In situ Preparations: Digoxigenin probes were made using standard protocol against the LacZ sequence (Cole et al. 2002) and against the Nrarp sequence (Shifley et al. 2008). Embryos were re-hydrated into PBT and then bleached with 6% Hydrogen peroxide in PBT for 1 hour at room temperature. They were then treated with 10 ug/mL Proteinase K at room temperature for 5-15 minutes, depending on size of the embryo. The embryos were then washed in fresh 2 mg/mL glycine in PBT for 10 minutes at room temperature. They were fixed in fresh 0.2% gluteraldehyde in 4% PFA for 20-30 minutes. The hindbrains were pierced and the embryos were treated with prehyb for 1 hour at 70° C. Embryos were incubated in hyb (prehyb

containing the probe) overnight at 70° C. After hybridization, the embyros were washed with 50% Formamide and 5X SSC pH 4.5 at 70° C and 50% Formamide and 2X SSC pH 4.5 at 65° C for three one hour washes each. Embryos were then incubated in 0.5 uL Anti-Digoxigenin-AP antibody, MABT, 2% BBR, and 20% heat inactivated sheep serum at 4° C over night. Post-antibody washes were performed with MABT and 2mM Levamisole. The next day, embryos were incubated in 5 mLs of detection solution containing NTMT, 2mM Levamisole, 35.3 uL NBT, and 13 uL BCIP at room temperature covered in foil until desired staining could be visualized. The embryos were then washed in PBT pH 5.5. They were post-fixed in 4% Paraformaldehyde and 0.1% Gluteraldehyde for 1 hour at room temperature. The embryos were then cleared by washing them in a 1:1 Glycerol:PBT solution for 1 hour and then stored in a 4:1 Glycerol:PBT solution.

Making Transgenic Mice

Creating the transgene: *Dll3* coding sequences were isolated from pKMV1 and subcloned into pBluescript (pDMW12). The *Mesp2* promoter was then isolated from pDMW4 and subcloned into pDMW12 (pDMW13). Digested pDMW13 containing *Dll3* attached to an *Mesp2* promoter was then ligated with Internal Ribosomal Entry Site (IRES) Bgeo 5', IRES Bgeo 3', and BSKS 3.0. Subcloning was accomplished using standard protocols.

Injecting and assaying transmission and expression: The transgene was prepared for insertion and wild type mouse tail DNA was spiked with the transgene using the standard protocol. Mouse embryos were injected with the transgenic DNA and they were assayed for expression of the transgene as adult mice using the DNA tail preparation protocol and PCR for pSEC169. We had 5 male and 2 female founders for pSEC169. These wild type mice were bred

to assay for transmission of pSEC169 to offspring using *in situ* hybridization for *Lac Z* and the *Lac Z* Staining of Embryos protocol.

Lac Z Staining of Embryos: Embryos were dissected into cold PBS and fixed in 1% formaldehyde, 0.2% gluteraldehyde, 2 mM MgCl2, 5mM EGTA (pH 8.0), and 0.02% NP-40 in PBS at 4°C for 30-90 minutes. Embryos were stained in the dark in 0.5 mL of 1 mg/mL X-gal, 5mM $K_3FE(CN)_6$, 5mM $K_4FE(CN)_6$, 2mM $MgCl_2$, 0.01% deoxycholic acid, and 0.02% NP-40 in PBS at room temperature overnight. Lastly, embryos were post-fixed in the same gluteraldehyde/formaldehyde fixing solution and stored in 70% EtOH at 4°C.

Skeletal Preparations

D+/pu; L+/+ x D+/+; L+/- cross: Embryos were harvested at 17.5 d.p.c. using standard protocols and genotyped for *Dll3* and *Lfng* using the Tail Preparation Protocol. Double and single heterozygotes and double knock outs were skinned and eviscerated. Skeletons were fixed in 100% EtOH for four days and then placed in acetone to remove fat for three days. Skeletons were then placed in a staining solution containing Alcian Blue, Alizarin red, glacial acetic acid and 70% EtOH for 10 days. The skeletons were cleared by placing them in 1% KOH in 20 % glycerol at 37°C overnight and then at room temperature until completely cleared. The skeletons were then placed in a clearing solution containing a 2:2:1 mix of EtOH:Glycerol:Benzyl Alcohol. Embryos were visualized under the microscope and pictures were taken with Axio Cam.

Primers Used

SC-320 Dll3	5’-CAGAAAGAGGTGGAGGTTGG-3’
SC-340 pSEC169	5’-CAGAATCCACACCTCTGCAA-3’
SC-342 pSEC169	5’-GGAAGGAGAAAAGCCAGGAT-3’
SC-355 Dll3	5’-GCCTCTTCTTCAGGGTCTGC-3’
SC-356 Dll3	5’-ACTCACCGGCCAAGCATC-3’
FNG322 Lfng	5’-GAGCACCAGGAGACAAGCC-3’
FNG325 Lfng	5’-AGAGTTCCTGAAGCGAGAG-3’
PGK3 Lfng	5’-CTTGTGTAGCGCCAAGTGC-3’
KT-53 pSEC169	5’-GGACAGCGTCTGAGACTTGA-3’
KT-54 pSEC169	5’-TCAGGTCGGAATTGAGGC-3’

V. References

Aulehla, A. On periodicity and directionality of somitogenesis. *Anat Embryol*. **211** (Suppl. 1), S3-8 (2006).

Barna, M. Plz regulates limb and axial skeletal patterning. *Nat. Gen.* **25:** 166-72 (2000).

Cole, S. Clock regulatory elements control cyclic expression of Lunatic fringe during somitogenesis. *Dev. Cell.* **3**: 75-84 (2002).

Dale, J. Periodic notch inhibititon by lunatic fringe underlies the chick segmentation clock. *Nature*. **421**: 275-8 (2003).

Dubrulle, J. Coupling segmentation to axis formation. *Development*. **131**: 5783-93 (2004).

Dunwoodie, S. Mouse *Dll3*: a novel divergent *Delta* gene which may complement the function of other *Deltta* homologues during early pattern formation in the mouse embryo. *Development*. **124**: 3065-3076 (1998).

Dunwoodie, S. Axial skeletal defects caused by mutation in the spondylocostal dysplasia/pudgy gene *Dll3* are associated with disruption of the segmentation clock within the presomitic mesoderm. *Development*. **129**, 1795-1805 (2002).

Evrard, Y. Lunatic fringe is an essential mediator of somite segmentation and patterning. *Nature*. **394:** 377-81 (1998).

Erol, B. Etiology of congenital scoliosis. University of Pennsylvania. *Orthopaedic Journal*. **15**: 37-42 (2002).

Geffers, I. Divergent functions and distinct localization of the Notch ligands DLL1 and DLL3 in vivo. J. *Cell Bio*. 178 (3), 465-76 (2007).

Gilbert, S. *Developmental Biology*. 8th ed. Sinauer, 2006.

Haraguchi, S. Transcriptional regulation of Mesp1 and Mesp2 genes: differential usage of enhancers during development. *Mech. Dev.* **108**: 59-69 (2001).

Kaufmann, M. *Atlas of Mouse Development*. Academic Press, 1992.

Kusumi, K. *Dll3* pudgy mutation differentially disrupts dynamic expression of somite genes. *Genesis*. **39**, 115-121 (2004).

Kusumi, K. The mouse pudgy mutation disrupts *Delta* homologue *Dll3* and initiation of early somite boundaries. *Nat. Genet.* **19**, 274-278 (1998).

Ladi, Ena. The divergent DSL ligand Dll3 does not activate Notch signaling but cell automously attenuates signaling induced by other DSL ligands. *J. Cell Bio*. **170**, 983-991 (2005).

Loomes, KM. Dll3 and Notch1 genetic interactions model axial segmental and craniofacial malformations of human birth defects. 236(10), 2943-2951 (2007).

Moloney, D. Fringe is a glycosyltransferase that modifies Notch. *Nature*. **406**: 369-375 (2000).

Morimoto, Mitsuru. The Mesp2 transcription factor establishes segmental borders by suppressing Notch activity. *Nature*. **435**: 354-9 (2005).

Nichols, J. Notch signaling--constantly on the move. *Traffic*. **8**, 959-69 (2007).

Nichols, J.T. DSL ligand endocytosis physically dissociates Notch 1 heterodimers before activating proteolysis can occur.

Saga, Y. Segmental border is defined by the key transcription factor Mesp2, by means of the suppression of Notch activity. *Dev. Dyn*. **236**: 1450-5 (2007).

Shifley, E. The vertebrate segmentation clock and its role in Skeletal Birth Defects. *Birth Defects*. **81**, 121-33 (2007).

Shifley, E. Oscillatory lunatic fringe activity is crucial for segmentation of the anterior but not the posterior skeleton. *Development*. **135**, 899-908 (2008).

Sparrow, D.B. Mutation of the *LUNATIC FRINGE* gene in humans causes Spondylocostal Dysostosis with a severe vertebral phenotype. *A.J. Hum. Gen.* **78**, 28-36 (2006).

Takahashi, Y. Mesp2 initiates somite segmentation through the Notch signaling pathway. *Nat. Genet*. **25**: 390-396.

Takahashi, Yu. Feedback loops comprising Dll1, Dll3 and Mesp2, and differential involvement of Psen1 are essential for rostrocaudal patterning of somites. *Development*. **18**: 4259-68 (2003).

Weinmaster, G. Modulation of Notch Signaling during Somitogenesis. *Cell Dev. Biol*. 19, 367-95 (2003).

Turnpenny, P.D. Novel mutations in *Dll3*, a somitogenesis gene encoding a ligand for the *Notch* signaling pathway, cause a consistent pattern of abnormal vertebral segmentation in spondylocostal dysostosis. *J. Med. Genet.* **40**, 333-339 (2003).

Yusuf, Faisal. The eventful somite: patterning, fate determination and cell division in the somite. *Anat Embryol*. **211** (Suppl. 1), S21-30 (2006).

Zhang, N. Segmentation defects of N pathway mutants and absence of a synergistic phenotype in lunatic fringe/radical fringe double mutant mice. *Genesis*. **33:** 21-28 (2002).